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(54) Titre : UTILISATION DE GALα(1,3)GALACTOSYLE TRANSFERASE DANS LES THERAPIES PAR GREFFE ENTRE DEUX ESPECES

(54) Title: USE OF PORCINE GAL α(1,3) GALACTOSYL TRANSFERASE IN XENOGRAFT THERAPIES

(57) Abrégé/Abstract:
DNA sequences encoding a porcine Galα(1,3) galactosyl transferase and clones containing such sequences are provided. The porcine Galα(1,3) galactosyl transferase produces the Galα(1,3) Gal epitope on the surfaces or porcine cells. This epitope is recognized by human anti-Galα(1,3) Gal antibodies which are responsible for hyperacute rejection of xenotransplanted pig cells, tissues and organs. Methods of reducing such hyperacute rejection are also provided.

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(57) Abstract

DNA sequences encoding a porcine Galα(1,3) galactosyl transferase and clones containing such sequences are provided. The porcine Galα(1,3) galactosyl transferase produces the Galα(1,3) Gal epitope on the surfaces or porcine cells. This epitope is recognized by human anti-Galα(1,3) Gal antibodies which are responsible for hyperacute rejection of xenotransplanted pig cells, tissues and organs. Methods of reducing such hyperacute rejection are also provided.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule comprising:
   (a) the nucleic acid sequence of SEQ ID NO: 1; or
   (b) an antisense sequence complementary to (a); or
   (c) both (a) and (b).

2. An isolated nucleic acid molecule comprising:
   (a) the nucleic acid sequence of SEQ ID NO:2; or
   (b) an antisense sequence complementary to (a); or
   (c) both (a) and (b).

3. Clone pPGT-4 having deposit designation number AGAL N94/9030.

4. Clone pPGT-2 having deposit designation number AGAL N94/9029.

5. Clone λPGT-g1 having deposit designation number AGAL N94/9027.

6. Clone λPGT-g5 having deposit designation number AGAL N94/9028.

7. A porcine cell comprising an inactivated porcine α(1,3) galactosyl transferase gene, said inactivated porcine α(1,3) galactosyl transferase gene comprising a wild type porcine α(1,3) galactosyl transferase sequence disrupted by a cloned mutant porcine α(1,3) galactosyl transferase sequence wherein the cloned mutant porcine α (1,3) galactosyl transferase sequence comprises a mutation of SEQ ID NO:1 wherein the mutation is selected from the

group consisting of a deletion, an insertion, a substitution, and an addition such that the cloned mutant porcine α(1,3) galactosyl transferase sequence does not encode a functional galactosyl transferase so that immune reaction of the cell with human antibodies reactive with Galα(1,3)Gal epitopes is avoided.

8. A porcine cell comprising an inactive porcine α(1,3) galactosyl transferase gene, said inactivated porcine α(1,3) galactosyl transferase gene comprising a wild type porcine α(1,3) galactosyl transferase sequence disrupted by a cloned mutant porcine α(1,3) galactosyl transferase sequence wherein the cloned mutant porcine α(1,3) galactosyl transferase sequence comprises a mutation of SEQ ID NO:2, wherein the mutation is selected from the group consisting of a deletion, an insertion, a substitution, and an addition such that the cloned mutant porcine α(1,3) galactosyl transferase sequence does not encode a functional galactosyl transferase so that immune reaction of the cell with human antibodies reactive with Galα(1,3)Gal epitopes is avoided.

Use of Porcine Gal α (1,3) galactosyl transferase in xenograft therapies

This invention relates to xenotransplantation (transplantation across species) and is particularly concerned with methods of alleviating xenotransplant rejection, maintenance of xenotransplanted tissue in an animal, nucleotide sequences useful in xenotransplant therapies, rejection resistant transgenic organs, and transgenic animals whose tissues are rejection-resistant on xenotransplantation.

The current shortage of tissues for human transplantation has led to recent close examination of xenografts as a possible source of organs. However, when tissues from non human-species are grafted to humans, hyperacute rejection occurs due to the existence of natural antibodies in human serum which react with antigens present in these species, with rejection occurring within 10-15 minutes of transplantation. This phenomenon depends, in general, on the presence of some or all of antibody, complement, neutrophils, platelets and other mediators of inflammation. In transplantation of vascularized organs between "discordant" species (those in which natural antibodies occur) the first cells to encounter natural antibodies are the endothelial cells

lining the blood vessels and it is likely that activation of these cells is induced by antibody binding to xenoantigens or other factors, leading to hyperacute rejection.

There is considerable uncertainty in the art concerning the nature of possible target xenoantigens on xenograft tissues. Platt et al (Transplantation 50:817-822,1990) and Yang et al (Transplant. Proc. 24:593-594, 1992) have identified a triad of glycoproteins of varying molecular weights as the major targets on the surface of pig endothelial cells. Other investigators (Holgersson et al, Transplant Proc 24:605-608, 1992) indicate glycolipids as key xenoantigens.

We have now found that xenograft rejection, particularly in the context of pig tissue, is associated with antibodies reactive with galactose in an $\alpha(1,3)$ linkage with galactose, (the Galα(1,3)Gal epitope) modulating the interaction between antibodies reactive with the Galα(1,3)Gal epitope of xenotransplant tissue effects rejection.

According to one aspect of the invention, there is provided an isolated nucleic acid molecule comprising:

(a) the nucleic acid sequence of SEQ ID NO: 1; or

(b) an antisense sequence complementary to (a); or

(c) both (a) and (b).

According to another aspect of the invention, there is provided an isolated nucleic acid molecule comprising:

(a) the nucleic acid sequence of SEQ ID NO:2; or

(b) an antisense sequence complementary to (a); or

(c) both (a) and (b).

According to a further aspect of the invention, there is provided a clone pPGT-4 having deposit

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designation number AGAL N94/9030.

According to another aspect of the invention, there is provided a clone pPGT-2 having deposit designation number AGAL N94/9029.

According to a further aspect of the invention, there is provided a clone λPGT-g1 having deposit designation number AGAL N94/9027.

According to another aspect of the invention, there is provided a clone λPGT-g5 having deposit designation number AGAL N94/9028.

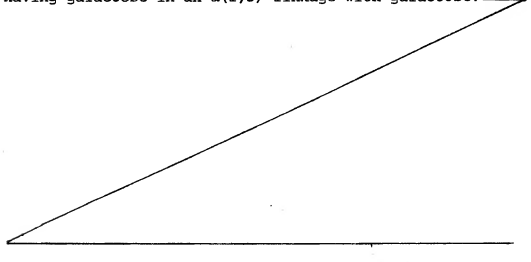
According to a further aspect of the invention, there is provided a porcine cell comprising an inactivated porcine α(1,3) galactosyl transferase gene, the inactivated porcine α(1,3) galactosyl transferase gene comprising a wild type porcine α(1,3) galactosyl transferase sequence disrupted by a cloned mutant porcine α(1,3) galactosyl transferase sequence wherein the cloned mutant porcine α (1,3) galctosyI transferase sequence comprises a mutation of SEQ ID NO:1 wherein the mutation is selected from the group consisting of a deletion, an insertion, a substitution, and an addition such that the cloned mutant porcine α(1,3) galactosyl transferase sequence does not encode a functional galactosyl transferase so that immune reaction of the cell with human antibodies reactive with Galα(1,3)Gal epitopes is avoided.

According to another aspect of the invention, there is provided a porcine cell comprising an inactive porcine α(1,3) galactosyl transferase gene, the inactivated porcine α(1,3) galactosyl transferase gene comprising a wild type porcine α(1,3) galactosyl transferase sequence

disrupted by a cloned mutant porcine α(1,3) galactosyl transferase sequence wherein the cloned mutant porcine α(1,3) galactosyl transferase sequence comprises a mutation of SEQ ID NO:2, wherein the mutation is selected from the group consisting of a deletion, an insertion, a substitution, and an addition such that the cloned mutant porcine α(1,3) galactosyl transferase sequence does not encode a functional galactosyl transferase so that immune reaction of the cell with human antibodies reactive with Galα(1,3)Gal epitopes is avoided.

According to a further aspect of the invention, there is provided a method for blocking human anti-Galα(1,3)Gal antibodies comprising changing the conformation of the antibody reactive site so as to reduce the affinity of the antibody for the Galα(1,3)Gal epitopes.

In accordance with the first aspect of this invention, there is provided a method of inhibiting xenotransplant rejection in an animal patient, comprising administering to the patient an effective amount of an antagonist of antibody binding to xenotransplant antigens having galactose in an α(1,3) linkage with galactose.



Another aspect of this invention relates to the maintenance of xenograft tissue in an animal, which comprises administering to the animal a graft rejection effective amount of an antagonist to antibodies which bind to the xenograft antigen epitope Galα(1,3)Gal.

In another aspect of this invention there is provided a method of inhibiting the binding of antibodies to the Galα(1,3)Gal epitope which comprises modulating the interaction between the antibodies and the epitope with an antagonist which blocks the binding of the antibodies to the Galα(1,3)Gal epitope.

Preferably the xenograft recipient is a human. Age is not a determining factor for xenograft transplantation although transplants in the elderly over 75 years would normally not be carried out. The invention is directed particularly to human transplantation with xenograft tissue.

Xenografted tissue is preferably of pig origin. Tissues from other mammals are also contemplated for use in this invention. Preferably the xenotransplanted tissue is in the form of an organ, for example, kidney, heart, lung or liver. Xenotransplant tissue may also be in the form of parts of organs, cell clusters, glands and the like. Examples include lenses, pancreatic islet cells, skin and corneal tissue. The nature of the xenotransplanted tissue is not of itself critical as any xenotransplanted tissue which expresses antigens having

Galα(1,3)Gal epitopes may be utilized in accordance with this invention.

The binding of antibody to the Galα(1,3)Gal epitope expressed on xenotransplanted tissue provokes rejection of the tissue by humoral as well as cell-mediated immune effects leading to tissue rejection in a very short time scale, such as less than one hour. Antagonists which antagonize the binding of antibodies to the Galα(1,3)Gal epitope block antibody binding and therefore inhibit xenotransplant rejection. Because antibody binding is blocked, immune responses which give rise to tissue rejection are prevented.

In accordance with a further aspect of this invention, there is provided an antagonist which modulates the interaction of antibodies directed against Galα(1,3)Gal.

Any antagonist capable of modulating the interaction between antibodies directed to the Galα(1,3)Gal linkage may be utilized in this invention. By reference to modulation, is meant blockage of antibody binding or decrease in affinity reactivity of antibodies for the Galα(1,3)Gal epitope. Various mechanisms may be associated with the blockage of antibody binding or decreased affinity of antibodies for their respective epitope. These include binding or association with the antibody reactive site and change of conformation of the antibody reactive site, such as by binding to residues associated with, adjacent to, or distanced from the

active site, which effect the conformation of the active site such that it is incapable of binding the Galα(1,3)Gal epitope or binds the epitope with reduced affinity. For example, in accordance with techniques well known in the art (see, for example, Coligan, et al., eds. Current Protocols In Immunology. John Wiley & Sons, New York, 1992; Harlow and Lane, Antibodies. A Laboratory Manual. Cold Spring Harbor Laboratory, New York, 1988; and Liddell and Cryer, A Practical Guide To Monoclonal Antibodies. John Wiley & Sons, Chichester, West Sussex, England, 1991), such a change of the conformation of the antibody reactive site can be achieved through the use of an anti-idiotypic antibody raised against the natural antibody or fragments thereof. As is also well known in the art, these anti-idiotypic antibodies may be modified to enhance their clinical usefulness, for example by enzymatic techniques such as preparing Fab' fragments, or by recombinant techniques such as preparing chimeric, humanized, or single chain antibodies.

This invention is not limited to any specific antagonist and any antagonist which is non-toxic and which modulates the interaction between antibodies specific for the Galα(1,3)Gal epitope may be used in this invention. Suitable examples of antagonists include D-galactose and melibiose, stachyose and methyl-α-D-galactopyranoside, D-galactosamine and derivatives thereof. The term derivatives encompasses, for example, any alkyl, alkoxy, alkylkoxy, aralkyl amine,

hydroxyl, nitro, heterocycle, sulphate and/or cycloalkyl substituents whether taken alone or in combination, which derivatives have antagonist activities. This may be assessed according to methods as herein described. Carbohydrate polymers containing one or more of the aforesaid carbohydrate moieties or derivatives may also be utilized in this invention.

The amount of antagonists which is effective to modulate interaction between antibodies reactive with Galα(1,3)Gal epitopes will vary depending upon a number of factors. These include the nature of the animal being treated, the nature of species of the transplanted tissue, the physical condition of the transplant recipient (age, weight, sex and health) and the like. In respect of human transplant recipients of tissue, for example from pigs, the amount of antagonists administered will generally depend upon the judgement of a consulting physician. As an example, a graft rejection effective amount of an antagonist in human subjects may be in the order of from 0.01mg to 1000gm per dose, more preferably 10mg to 500mg, more preferably 50mg to 300mg, and still more preferably 50mg to 200mg per dose.

The schedule of administration of antagonists to inhibit rejection and maintain xenografts will depend upon varying factors as mentioned above. Varying dosage regimes may be contemplated, such as daily, weekly, monthly or the like.

The mode of administration of antagonists and dosage forms thereof are not critical to this invention. Antagonists may be administered parenterally (intravenous, intramuscular or intraorgan injection), orally, transdermally, or by vaginal or anal routes, or by other routes of administration, as are well known in the art. Antagonists may be in solid or liquid form and would generally include pharmaceutically acceptable or veterinarially acceptable excipients and/or carriers. Examples of dosage forms which may be used in this invention are those well known in the art as mentioned previously such as described in Remington's Pharmaceutical Sciences (Mack Publishing Company, 10th Edition.

In still another aspect of this invention, there is provided nucleotide sequences encoding α(1,3) galactosyl transferase and mutants thereof. Preferably, the nucleotide sequence encodes pig α(1,3) galactosyl transferase.

Nucleotide sequences may be in the form of DNA, RNA or mixtures thereof. Nucleotide sequences or isolated nucleic acids may be inserted into replicating DNA, RNA or DNA/RNA vectors as are well known in the art, such as plasmids, viral vectors, and the like (Sambrook et al, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY, Second Edition 1989).

Nucleotide sequences encoding α(1,3) galactosyl transferase may include promoters, enhancers and other

regulatory sequences necessary for expression, transcription and translation. Vectors encoding such sequences may include restriction enzyme sites for the insertion of additional genes and/or selection markers, as well as elements necessary for propagation and maintenance of vectors within cells.

Mutants of nucleotide sequences encoding α(1,3)galactosyl transferase are particularly preferred as they may be used in homologous recombination techniques as are well known in the art (Capecchi M R, Altering the Genome by Homologous Recombination, Science 244:1288-1292, 1989; Merlino G T, Transgenic Animals in Biomedical research, FASEB J 5:2996-3001, 1991; Cosgrove et al, Mice Lacking MHC Class II Molecules, Cell 66:1051-1066, 1991; Zijlstra et al, Germ-line Transmission of a disrupted B2-microglobulin gene produced by homologous recombination in embryonic stem cells, Nature 342:435, 1989) for the inactivation of wild type α(1,3) galactosyl transferase genes.

Mutant α(1,3) galactosyl transferase nucleotide sequences include nucleotide deletions, insertions, substitutions and additions to wild type α(1,3) galactosyl transferase such that the resultant mutant does not encode a functional galactosyl transferase. These nucleotide sequences may be utilized in homologous recombination techniques. In such techniques, mutant sequences are recombined with wild type genomic sequences in stem cells, ova or newly fertilized cells comprising

from 1 to about 500 cells. Nucleotide sequences utilized in homologous recombination may be in the form of isolated nucleic acids sequences or in the context of vectors. Recombination is a random event and on recombination, destruction of the functional gene takes place.

Transgenic animals produced by homologous recombination and other such techniques to destroy wild type gene function are included within this invention, as are organs derived therefrom. By way of example, transgenic pigs may be produced utilizing homologous recombination techniques to produce a transgenic animal having non-functional α(1-3) galactosyl transferase genomic sequences. Tissues derived from such transgenic animals may then be utilized in xenotransplantation into human patients with the avoidance of immune reaction between circulating human antibodies reactive with Galα(1-3)Gal epitopes. Such transplants are contemplated to be well tolerated by transplant recipients. Whilst transplanted tissue may comprise other antigens which provoke immune reaction beyond those associated with Galα(1-3)Gal epitopes, removing the major source of the immune reaction with such transplanted tissues should lead to xenotransplants being relatively well tolerated in conjunction with standard rejection therapy (treatment with immune suppressants such as cyclosporin).

This invention will now be described with reference to the following non-limiting Figures and Examples.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Figure 1A shows titer of pooled human serum before and after absorption. Titer obtained by hemagglutination on RBC (hatched bars) and rosetting assay on PBL (open bars) and spleen cells (solid bars). Absorption studies demonstrated that the same xeno antigens were present on all of these tissues (Figure 1 and Figure 2), as absorption with RBC, spleen cells or PBL, removed reactivity for the other cells (Figure 1A and Figure 2). Absorption of the serum pool with EC, while removing all of the EC reactive antibodies (Figure 2A), completely removed all PBL reactive antibodies and almost all the RBC hemagglutinating antibodies (titer fell from 1/128 to 1/2) (Figure 1A). Absorption with RBC removed 75% (Figure 2B) and spleen cells all (Figure 2C) of the endothelial cell (CE) reactive antibodies shown by flow cytometry. Thus, common epitopes are present on pig red cells, PBL, spleen and endothelial cells. Serum absorbed with EC was not tested on PBL or spleen cells. Figure 1B -- see Figure 3.

Figure 2: Testing of pig EC with pooled human serum before and after absorption. In each panel EC tested with absorbed serum (dotted line) or non absorbed serum (solid line). Serum absorbed with EC (panel A), RBC (panel B) or spleen cells (panel C). Binding of human antibody was detected using sheep anti-human IgM and analysis by flow cytometry.

Figure 3: Hemagglutination titer of treated and untreated human serum. Untreated human serum (A); protein-A non binding immunoglobulin (B); protein-A eluted immunoglobulin (C); serum treated with 2-mercaptoethanol (D). Figure 1B shows the same data with the addition of data obtained using a high molecular weight immunoglobulin fraction. Figure 1B: Untreated human serum (A); protein-A non binding immunoglobulin (B); high molecular weight fraction (C); protein-A eluted immunoglobulin (D); serum treated with 2-mercaptoethanol (E).

Figure 4: Carbohydrate inhibition of hemagglutination of normal human serum. Human serum was titered in the presence of 300mM solutions of carbohydrates.

Figure 5: Concentration of carbohydrate giving 50% inhibition of hemagglutination titer of normal human serum. Only carbohydrates inhibiting hemagglutination in Figure 4 were used in this experiment, with glucose and methyl-β-galactopyranoside as negative controls.

Figure 6: Hemagglutination titer of human serum on pig RBC pre and post absorption on a melibiose column. Human serum was absorbed with equal volumes of melibiose-sepharose (solid bars) or sepharose (open bars), a number of times as indicated in the figure axis.

Figure 7: Southern blot of pig genomic DNA probed with the cDNA insert of clone pPGT-4.

BRIEF DESCRIPTION OF THE SEQUENCE LISTINGS

SEQ ID NO:1 Partial nucleotide and predicted amino acid sequence of the pig Galα(1,3) transferase.

SEQ ID NO:2 Complete nucleotide and predicted amino acid sequence of the pig Galα(1,3) transferase.

SEQ ID NO:3 Nucleotide sequence for PCR primer αGT-1.

SEQ ID NO:4 Nucleotide sequence for PCR primer αGT-2.

With regard to SEQ ID NOS:1-2, it should be noted that the present invention is not limited to the specific sequences shown, but, in addition to the mutations discussed above, also includes changes that are found as naturally occurring allelic variants of the porcine Gal α(1,3) galactosyl transferase gene, as well as nucleic acid mutations which do not change the amino acid sequences set forth in these sequences, e.g., third nucleotide changes in degenerate codons.

EXAMPLE 1

Materials and Methods

Cells. Pig cells and tissues were obtained from an abattoir from freshly slaughtered animals. Whole blood was centrifuged at 800g, and erythrocytes (RBC) obtained and were washed three times in phosphate buffered saline (PBS); pig peripheral blood lymphocytes (PBL) were isolated by density gradient centrifugation using ISOPAQUE FICOLL (Vaughan et al, (1983) Transplantation 36:446-450). Pig splenocytes were obtained from whole

spleen by teasing tissue through a sieve to give a single cell suspension. Endothelial cell (EC) cultures were established after treatment of sterile pig aorta with Collagenase Type 4 (Worthington Biochemical Corporation, New Jersey) and the isolated cells were grown in Dulbecco's modified Eagles medium (DMEM) (ICN Biomedicals Australasia Pty Ltd, Seven Hills, NSW) on gelatin coated plates at 37°C. The endothelial origin of EC cultures was verified using rabbit anti human von Willebrand factor antibody (Dako A/S, Copenhagen) and indirect immunofluorescence. COS cells used were maintained in fully supplemented DMEM medium.

Antibodies. Human serum was obtained from a panel of normal volunteers, heat inactivated and pooled before use. The mAb HuLy-m3 (CD48), was used as a negative control (Vaughan Supra). Equal volumes of human serum and 5 to 200mM 2-mercaptoethanol were incubated at 37°C for one hour to destroy IgM.

Absorptions. Pooled serum was absorbed with equal volumes of washed, packed cells for 15 minutes at 37°C, for 15 minutes at 4°C, serum obtained and the procedure repeated three times. For the absorption with melibiose-agarose (Sigma, St Louis, MO) and sepharose™ (Pharmacia LKB Biotechnology, Uppsala, Sweden), equal volumes packed beads and serum were incubated at 37°C for 16 hours, the beads removed by centrifugation, and the absorption repeated several times.

Serological Assays. a) Hemagglutination: 50µl of 0.1% pig RBC were added to 50µl of human serum in 96 well plates, incubated at 37°C for 30 minutes, room temperature for 30 minutes and on ice for 60 minutes prior to both macroscopic and microscopic evaluation of hemagglutination; b) Rosetting: Sheep anti human IgG was coupled to sheep RBC with chromic chloride and used in a rosetting assay (Parish et al (1978) J Immunol. Methods 20:173-183); c) Cytofluorographic analysis was performed on FACScan™ (Becton Dickinson, San Jose, CA) (Vaughan et al (1991) Immunogenetics 33:113-117); d) Indirect immunofluorescence was performed on cell monolayers in 6 well tissue culture plates using fluoresceinated sheep anti human IgM or IgG (Silenus Laboratories Pty Ltd, Hawthorn, Victoria, Australia) (Vaughan Supra).

Sugar Inhibitions. Two types of sugar inhibition assays were performed: a) 50µl of sugars (300mM in PBS) were added to 50µl of doubling dilutions of human serum in 96 well plates, incubated overnight at 46°C and then 50µl of 0.1% pig RBC added and the hemagglutination assay performed; b) Human serum, diluted in PBS at one dilution less than that of the 50% hemagglutination titer, was added to 50µl of doubling dilutions of sugars (starting at 300mM) and incubated overnight at 4°C, after which 50µl of 0.1% pig RBC was added and the hemagglutination assay performed.

Murine Gal α(1-3) Transferase cDNA construct. A cDNA clone, encoding the mouse α(1,3)galactosyl

transferase was produced using the published sequence of this transferase (Larsen et al (1989) J Biol. Chem 264:14290-14297) and the polymerase chain reaction (PCR) technique. Briefly two oligonucleotides were synthesized; αGT-1 (5'-GAATTCAAGC TTATGATCAC TATGCTTCAA G-3') which is the sense oligonucleotide encoding the first six amino acids of the mature αGT and contains a HindIII restriction site, and αGT-2 (5'-GAATTCCTGC AGTCAGACAT TATTCTAAC-3') which is the anti-sense oligonucleotide encoding the last 5 amino acids of the mature αGT and the in phase termination codon and contains a PstI restriction site. This oligonucleotide pair was used to amplify a 1185 bp fragment from a C57BL/6 spleen cell cDNA library (Sandrin et al (1992) J Immunol. 194:1636-1641). The 1185 bp fragment was purified from a Low Gelling point agarose gel, digested with HindIII and PstI (Pharmacia) restriction endonucleases, and directionally cloned into HindIII/PstI digested CDM8 vector (Seed B (1987) Nature 329:840 842) using T4 ligase (Pharmacia). The product of the ligation was used to transform MC1061/p3, and DNA prepared from resultant colonies for further examination. One plasmid (pαGT-3) having the 1185 bp fragment was selected for further studies. Plasmid DNA was prepared, sequenced to confirm the correct DNA sequence, and used for COS cells transfection experiments using DEAE/Dextran (Vaughan et al (1991) Immunogenetics 33: 113-117; Sandrin et al

(1992) J Immunol. 194:1636-1641, Seed B (1987) Nature 329:840-842).

EXAMPLE 2

Human Anti-pig Antibodies Detect

Epitopes Present on Different Cells

To establish that human serum contains antibodies to pig cells which are predominantly of the IgM class, a pool of human serum was made (from 10 donors) and found to contain antibodies which reacted with pig red cells (by hemagglutination); pig lymphocytes (rosetting assay and flow cytometry); pig spleen cells (rosetting); and pig endothelial cells (flow cytometry) (Figures 1 and 2). Absorption studies demonstrated that the same xeno antigens were present on all of these tissues (Figure 1 and Figure 2), as absorption with RBC, spleen cells or PBL, removed reactivity for the other cells (Figure 1A and Figure 2). Absorption of the serum pool with EC, while removing all of the EC reactive antibodies (Figure 2a), completely removed all PBL reactive antibodies and almost all the RBC hemagglutinating antibodies (titer fell from 1/128 to 1/2) (Figure 1A). Absorption with RBC removed 75% (Figure 2B) and spleen cells all (Figure 2C) of the EC reactive antibodies shown by flow cytometry. Thus, common epitopes are present on pig red cells, PBL, spleen and endothelial cells.

Most of the activity in the serum pool was due to IgM rather than IgG antibodies as indicated by the inability of a protein A-sepharose column, which does not

bind IgM, to alter the titer of the serum after passage through the column (Figure 3), and IgG antibodies eluted from the protein A-sepharose column reacted only weakly with RBC (Figure 3). Furthermore, treatment of the serum with 2-mercaptoethanol, which destroys IgM but leaves IgG intact, led to a complete loss of antibody activity (Figure 3). When the serum was fractionated by SEPHACRYL™ gel chromatography, the high molecular weight fractions (IgM) were reactive with RBC, whereas the low molecular weight fractions (IgG) were not (data not shown). Thus the different pig cells carry similar epitopes, all reacted with IgM antibodies and in our assays there was little IgG activity found in the human serum for pig cells.

EXAMPLE 3

Human Anti-pig Antibodies React Predominantly With Terminal Galactose Residues

The ability of different carbohydrates to inhibit the hemagglutination reaction (Figure 4) was examined. Of the sugars tested, inhibition as measured by a decrease in titer, was observed with 300mM galactose, methyl-α-D-galactopyranoside, melibiose and stachyose, all of which decreased the titer of the serum pool by 75% (Figure 4); and with 300mM D-galactosamine, for which a 50% decrease in titer was observed (Figure 4). None of the other monosaccharides tested (listed in the figure legend) had any effect on hemagglutination titer (Figure 4). These studies demonstrated that galactose is the

part of the epitope, as both melibiose and stachyose have terminal galactose residues. It is of interest to note the difference in the ability of galactose in the α(methyl-α-D-galactopyranoside, melibiose and stachyose) but not β(methyl-β-D-galactopyranoside) configuration to inhibit the serum.

The relative avidity of the antibodies for the sugars which inhibited agglutination was estimated from the concentration of sugar giving 50% inhibition of the agglutination titer (Figure 5). Both D-galactose and melibiose achieved this inhibition at <1.5mM, stachyose and methyl-α-D-galactopyranoside at 4.7mM and D-galactosamine at 18.7mM (Figure 5). By contrast, D-glucose and methyl-β-D-galactopyranoside had no effect even at 300mM concentration. Thus D-galactose is an important part of the epitope, as it is a potent inhibitor of the xenoantibodies at low concentration (<1.1 5mM); the ability of methyl-α-D-galactopyranoside to inhibit agglutination at low concentrations (<1.15mM), compared with the failure of methyl-β-D-galactopyranoside (300mM) to inhibit, demonstrates that the galactose residue (which is likely to be a terminal sugar) is in an α-linkage rather than a β-linkage with the subterminal residue. The results obtained with melibiose (Galα(1,6)Glc) and stachyose (Galα(1,6)Galα(1,6)Glcβ(1,2)Fru), which have α-linked terminal galactose residues, are in accord with this

conclusion. The inhibition of hemagglutination observed with galactosamine, which has an additional amine side chain on galactose, (50% inhibition of titer at 18.7mM) could be due to a second carbohydrate involved in the epitope, or a lower affinity of the xenoantibodies for this sugar.

To further examine the reaction with galactose, the serum pool was absorbed four times with equal volumes of packed melibiose sepharose or with sepharose as the control (Figure 6), one absorption with melibiose-sepharose decreased the titer of the antibody from 1/32 to 1/4, and two sequential absorptions decreased the titer further to 1/2 (Figure 6). This absorption was specific for melibiose, as using sepharose beads had no effect (Figure 6). Thus the majority of the antibody (=94%) reactive with xenoantigens reacts with galactose in an α-linkage.

EXAMPLE 4

Human Anti-Pig Antibodies React with COS Cells After Transfection with α(1,3) Galactosyl Transferase

The cDNA coding for the α(1,3)galactosyl transferase which transfers a terminal galactose residue with an α(1,3) linkage to a subterminal galactose has been cloned for both mouse (Larsen et al (1989) J Biol Chem 264:14290-14297) and ox (Joziasse et al (1989) J Biol Chem 264:14290-14297). Using this data we used transfection experiments to determine the role of the Galα(1,3)Gal epitope in isolation of others. The mouse

transferase was isolated from a cDNA library using the PCR technique, and the PCR product was directionally cloned into the CDM8 vector for expression studies in COS cells. The cDNA insert was sequenced in both directions and shown to be identical to the published nucleotide sequence (Larsen et al (1989) J Biol Chem 264:14290-14297). COS cells, derived from Old World Monkeys, were chosen as they do not react with human serum nor with the IB-4 lectin (which is specific for the Galα(1,3)Gal epitope) (Table 1). After transfection of COS cells with the α(1,3)galactosyl transferase, the Galα(1,3)Gal epitope was detected on the cell surface by binding of the IB-4 lectin (Table 1); these cells were also strongly reactive with the serum pool. Absorbing the human sera with pig RBC removed the reactivity for Galα(1,3)Gal⁺COS cells, (Table 1). Passage of the serum over a protein-A sepharose column had no effect on the reactivity of the serum for Galα(1,3)Gal⁺COS cells, when using an FITC conjugated sheep anti-human IgM as the second antibody (this was reflected in the same number of reactive cells, the intensity of staining and the titer of the serum (Table 1)). In contrast to this, eluted antibodies reacted only weakly with the Galα(1,3)Gal⁺COS cells, and this reaction was only observed when using FITC conjugated sheep anti-human IgG or FITC conjugated sheep anti-human Ig, but not FITC conjugated sheep anti human IgM (Table 1). Thus human serum has IgM antibodies to the Galα(1,3)Gal epitope which was expressed on

Galα(1,3)Gal⁺COS cells. The reaction of the serum with Galα(1,3)Gal⁺COS cells is specific and not due to the transfection procedure as CD48⁺ COS cells were not reactive with either the serum nor the IB-4 lectin (Table 1). Furthermore, the reactivity for both pig RBC (as detected by hemagglutination) and EC (as detected by FACS analysis) could be removed by absorption with Galα(1,3)Gal⁺COS cells but not untransfected COS cells. Thus human serum pool contains IgM antibodies reactive with the Galα(1,3)Gal epitope.

The level of antibodies in human serum reactive with the Galα(1,3)Gal epitope can be used to determine the propensity of a patient to hyperacutely reject a porcine xenotransplant. In addition, the level of such antibodies can be used to determine the amount of antibody antagonist that should be administered to a patient prior to such xenotransplantation.

The level of these antibodies can be effectively determined using the transfected and untransfected COS cells described above as matched Galα(1,3)Gal⁺ and Galα(1,3)Gal⁻ absorbants, followed by a measurement of the reactivity of the absorbed serum for pig RBC and/or EC. Higher levels of serum antibody will result in a larger difference in reactivity of the serum absorbed against the Galα(1,3)Gal⁺ absorbant versus that absorbed against the Galα(1,3)Gal⁻ absorbant. Cells from other species, e.g., human cells, can be used in such an assay. Also, rather than using a DNA sequence encoding the murine

transferase, a DNA sequence encoding the porcine transferase (see Example 5) can be used. Such a porcine transferase is preferred since there may be differences in the action of the murine and porcine transferases, e.g., altered sensitivity to the macromolecular environment of the galactose substrate of the enzyme, and for a porcine xenotransplantation, it is the level of antibodies against the Galα(1,3)Gal epitope in the porcine macromolecular environment that is of interest.

In addition to the foregoing, the transfected Galα(1,3)Gal$^+$ cells described above can also be used as absorbants to remove anti-Galα(1,3)Gal antibodies from human serum, e.g., by binding such cells to a solid support and passing the serum over the immobilized cells.

EXAMPLE 5

Cloning of Porcine α(1,3) Galactosyl Transferase

Utilizing the murine cDNA clone for the α(1,3) galactosyl transferase as a hybridization probe we have cloned the pig α(1,3) galactosyl transferase from a λGT11 pig spleen cDNA library (Clontech Laboratories, Palo Alto, CA) according to standard methods as described in Sambrook et al (supra). This clone, pPGT-4, has been deposited with the AGAL and assigned accession number N94/9030. SEQ ID NO:1 shows a partial nucleotide sequence and predicted amino acid sequence of pig Galα(1,3) transferase as determined by sequencing of clone pPGT-4. The sequence shown is incomplete at the 5' end.

Utilizing the cDNA insert of the pPGT-4 clone as a hybridization probe we have also cloned the 5' end of the pig α(1,3) galactosyl transferase from a 5' STRECH pig liver cDNA library in λgt10, according to standard methods as described in Sambrook et al (supra). The insert was obtained by the PCR technique using a λ oligonucleotide, and an oligonucleotide made to the pig sequence. This PCR product was subcloned into SmaI cut pBLUESCRIPT KS*. This clone, pPGT-2, has been deposited with the AGAL and assigned accession number N94/9029.

SEQ ID NO:2 shows a complete nucleotide sequence and predicted amino acid sequence of pig Galα(1,3) transferase as determined by sequencing of clones pPGT-4 and pPGT-2. The pig transferase has high sequence homology with both the murine and bovine α(1,3) galactosyl transferase genes.

Both the partial and complete cDNA sequences of SEQ ID NOS:1-2 can be used in the xenotransplant therapies discussed above. For example, using techniques well known in the art, all or a part of any of the nucleotide sequences of SEQ ID NOS:1-2, when inserted into replicating DNA, RNA or DNA/RNA vectors, can be used to reduce the expression of the Galα(1,3) transferase in porcine cells by directing the expression of anti-sense RNAs in transgenic cells or animals. See, for example, Biotechniques, 6(10):958-976, 1988.

In addition, as illustrated in the following example, the sequences of SEQ ID NOS:1-2 can be used as hybridization probes for the characterization and isolation of genomic clones encoding the porcine Galα(1,3) transferase. Mutants of the genomic nucleotide sequence, in turn, can be used in homologous recombination techniques of the types described above so that destruction of the functional gene takes place in porcine cells.

EXAMPLE 6

Characterization and Isolation of the Porcine Gene Encoding α(1,3) Galactosyl Transferase

Genomic DNA prepared from pig spleen tissue was digested with EcoR1, BamH1, Pst1, HindIII, Kpn1 and BstEII, electrophoresed on a 0.8% agarose gel and transferred to a nylon filter, the final wash was at 65°C in 0.1x SSC, 0.1% SDS. As shown in Figure 7, the genomic Southern blot demonstrated a simple pattern suggesting that the gene exists as a single copy with a genomic size of ~25kb.

Utilizing the cDNA insert of the pPGT-4 clone as a hybridization probe, we have cloned the porcine α(1,3) galactosyl transferase gene from a pig genomic DNA EMBL library (Clontech Laboratories, Inc., Palo Alto, CA) according to standard methods as described in Sambrook et al (supra). This cloning has resulted in the isolation of two lambda phage clones, λPGT-g1 and λPGT-g5 that

contain different regions of the porcine transferase gene.

As discussed above, the gene for the α(1,3) galactosyl transferase can be used to effect targeted destruction of the native gene for this enzyme using homologous recombination technology. In accordance with the conventional techniques used in this art, such gene knockout is performed using fragments obtained from genomic clones of the type provided by this example. The gene destruction can be performed in somatic or stem cells (Capecchi, 1989, supra). Because such genetically engineered cells do not produce the Galα(1,3)Gal epitope, they and their progeny are less likely to induce hyperacute rejection in humans and are thus suitable for xenotransplantation into human patients.

EXAMPLE 7

Production of Anti-idiotypic Antibodies Against Human Anti-Galα(1,3)Gal Antibodies

Polyclonal anti-idiotypic antibodies against human anti-Galα(1,3)Gal antibodies are prepared following the procedures of Coligan, et al., 1992, supra; Harlow and Lane, 1988, supra; and Liddell and Cryer, 1991, supra. Human anti-Galα(1,3)Gal antibodies are absorbed from pooled human serum onto immobilized melibiose (melibiose-sepharose or melibiose-agarose) as described above in Example 3. The antibodies are eluted using standard methods, such as, high or low pH, high salt, and/or chaotropic agents. Fab' fragments are prepared following

dialysis into an appropriate buffer. The Fab' fragments are used to immunize rabbits, goats, or other suitable animals, along with conventional adjuvants.

The resulting polyclonal antisera are tested for their ability to change the conformation of the human antibody reactive site so as to reduce its affinity for the Galα(1,3)Gal epitope. Those sera that produce such reduced affinity constitute the desired anti-idiotypic antibodies.

Monoclonal antibodies are produced using the same Fab' fragments as antigens to immunize appropriate strains of mice. Hybridomas are prepared by fusing spleen cells from such immunized mice with murine myeloma cells. Supernatants are tested for antibodies having the ability to change the conformation of the human antibody reactive site so as to reduce its affinity for the Galα(1,3)Gal epitope. Those antibodies that produce such reduced affinity constitute the desired monoclonal anti-idiotypic antibodies.

The finding that the majority of xenoreactive IgM is directed to the enzymatic product of the single transferase raises the possibility of producing transgenic pigs lacking the epitope, by targeted destruction of the α(1,3) galactosyl transferase genes using homologous recombination technology. Such genetically modified pigs could be used for transplantation. The destruction of the gene is likely to have no deleterious effect on the pig - humans live

normally in its absence.

This invention has been described by way of example only and is in no way limited by the specific examples herewith.

DEPOSITS

Clones pPGT-4, pPGT-2, λPGT-g1, and λPGT-g5, discussed above, have been deposited with the Australian Government Analytical Laboratories, (AGAL), 1 Suakin Street, Pymble, N.S.W. 2073, Australia, and have been assigned the designations N94/9030, N94/9029, N94/9027, and N94/9028, respectively. These deposits were made under the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure (1977). These deposits were made on March 11, 1994.

TABLE 1

Serology On Transfected COS Cells

Serum	Target	Reaction[1]
NHS	GT*COS	+++
NHS abs RBC	GT*COS	-
NHS Tx 2-ME	GT*COS	-
NHS abs Protein A	GT*COS	+++[2]
NHS Eluted Protein A	GT*COS	+[3]
CD48	GT*COS	-
NHS	CD48*COS	-
CD48	CD48*COS	+++
NHS	COS	-
CD48	COS	-
IB4[4]	GT*COS	+++
IB4	CD48*COS	-
IB4	COS	-

[1] Reactivity detected by indirect immunofluorescence using FITC conjugated sheep anti-human Ig or FITC conjugated sheep anti-mouse Ig unless otherwise stated.

[2] No difference in titer was observed when tested with FITC conjugated sheep anti-human IgM.

[3] Reaction detected on protein A purified immunoglobulin using FITC conjugated sheep anti-human Ig or FITC conjugated sheep anti-human IgG, but not with FITC conjugated sheep anti-human IgM.

[4] Reactivity detected using FITC conjugated IB4 lectin.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The Austin Research Institute

(ii) TITLE OF INVENTION: XENOTRANSPLANTATION THERAPIES

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Peter A. Stearne

(B) STREET: Level 10, 10 Barrack Street

(C) CITY: Sydney

(D) STATE: New South Wales

(E) COUNTRY: Australia

(F) Postal Code 2001

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Peter A. Stearne

(C) REFERENCE/DOCKET NUMBER: 462552/pas

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 612 262 2611

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1353 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA to mRNA

(A) DESCRIPTION: galactosyl transferase, 3' clone

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Sus scrofa

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GTA CCG AGC TCG AAT TCC GCA AGC CAG TCA CCA CAA GCC ATG      42
Val Pro Ser Ser Asn Ser Ala Ser Gln Ser Pro Gln Ala Met
                50                  55                  60

ACT GAC CCA TGT TCC CCC AGA CTG TCG TAC CTT AGC AAA GCC      84
Thr Asp Pro Cys Ser Pro Arg Leu Ser Tyr Leu Ser Lys Ala
                65                  70

ATC CTG ACT CTA TGT TTT GTC ACC AGG AAA CCC CCA GAG GTC      126
Ile Leu Thr Leu Cys Phe Val Thr Arg Lys Pro Pro Glu Val
 75                  80                  85

GTG ACC ATA ACC AGA TGG AAG GCT CCA GTG GTA TGG GAA GGC      168
Val Thr Ile Thr Arg Trp Lys Ala Pro Val Val Trp Glu Gly
 90                  95                  100

ACT TAC AAC AGA GCC GTC TTA GAT AAT TAT TAT GCC AAA CAG      210
Thr Tyr Asn Arg Ala Val Leu Asp Asn Tyr Tyr Ala Lys Gln
        105                 110                 115

AAA ATT ACC GTG GGC TTG ACG GTT TTT GCT GTC GGA AGA TAC      252
Lys Ile Thr Val Gly Leu Thr Val Phe Ala Val Gly Arg Tyr
        120                 125                 130

ATT GAG CAT TAC TTG GAG GAG TTC TTA ATA TCT GCA AAT ACA      294
Ile Glu His Tyr Leu Glu Glu Phe Leu Ile Ser Ala Asn Thr
                135                 140

TAC TTC ATG GTT GGC CAC AAA GTC ATC TTT TAC ATC ATG GTG      336
Tyr Phe Met Val Gly His Lys Val Ile Phe Tyr Ile Met Val
145                 150                 155

GAC GAT ATC TCC AGG ATG CCT TTG ATA GAG CTG GGT CCT CTG      378
Asp Asp Ile Ser Arg Met Pro Leu Ile Glu Leu Gly Pro Leu
    160                 165                 170

CGT TCC TTT AAA GTG TTT GAG ATC AAG TCC GAG AAG AGG TGG      420
Arg Ser Phe Lys Val Phe Glu Ile Lys Ser Glu Lys Arg Trp
        175                 180                 185

CAA GAC ATC AGC ATG ATG CGC ATG AAG ACC ATC GGG GAG CAC      462
Gln Asp Ile Ser Met Met Arg Met Lys Thr Ile Gly Glu His
        190                 195                 200

ATC CTG GCC CAC ATC CAG CAC GAG GTG GAC TTC CTC TTC TGC      504
Ile Leu Ala His Ile Gln His Glu Val Asp Phe Leu Phe Cys
                205                 210
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ATT GAC GTG GAT CAG GTC TTC CAA AAC AAC TTT GGG GTG GAG   546
Ile Asp Val Asp Gln Val Phe Gln Asn Asn Phe Gly Val Glu
215                 220                 225

ACC CTG GGC CAG TCG GTC GCT CAG CTA CAG GCC TGG TGG TAC   588
Thr Leu Gly Gln Ser Val Ala Gln Leu Gln Ala Trp Trp Tyr
    230                 325                 240

AAG GCA CAT CCT GAC GAG TTC ACC TAC GAG CGG CCG AAG GAG   630
Lys Ala His Pro Asp Glu Phe Thr Tyr Glu Arg Pro Lys Glu
        245                 250                 255

TCC GCA GCC TAC ATT CCG TTT CGC CAG GGG GAT TTT TAT TAC   672
Ser Ala Ala Tyr Ile Pro Phe Arg Gln Gly Asp Phe Tyr Tyr
            260                 265                 270

CAC GCA GCC ATT TTG GGG GGA ACA CCC ACT CAG GTT CTA AAC   714
His Ala Ala Ile Leu Gly Gly Thr Pro Thr Gln Val Leu Asn
                275                 280

ATC ACT CAG GAG TGC TTC AAG GGA ATC CTC CAG GAC AAG GAA   756
Ile Thr Gln Glu Cys Phe Lys Gly Ile Leu Gln Asp Lys Glu
285                 290                 295

AAT GAC ATA GAA GCC GAG TGG CAT GAT GAA AGC GGG CTA AAC   798
Asn Asp Ile Glu Ala Glu Trp His Asp Glu Ser Gly Leu Asn
    300                 305                 310

AAG TAT TTC CTT CTC AAC AAA CCC ACT AAA ATC TTA TCC CCA   840
Lys Tyr Phe Leu Leu Asn Lys Pro Thr Lys Ile Leu Ser Pro
        315                 320                 325

GAA TAC TGC TGG GAT TAT CAT ATA GGC ATG TCT GTG GAT ATT   882
Glu Tyr Cys Trp Asp Tyr His Ile Gly Met Ser Val Asp Ile
            330                 335                 340

AGG ATT GTC AAG GGG GCT TGG CAG AAA AAA GAG TAT AAT TTG   924
Arg Ile Val Lys Gly Ala Trp Gln Lys Lys Glu Tyr Asn Leu
                345                 350

GTT AGA AAT AAC ATC TGACTTTAAA TTGTGCCAGC AGTTTTCTGA      969
Val Arg Asn Asn Ile
355

ATTTGAAAGA GTATTACTCT GGCTACTTCC TCAGAGAAGT AGCACTTAAT    1019

TTTAACTTTT CAAAAAATAC TAACAAAATA CCAACACAGT AAGTACATAT    1069

TATTCTTCCT TGCAACTTTG AGCCTTGTCA AATGGGAGAA TGACTCTGTA    1119

GTAATCAGAT GTAAATTCCC AATGATTTCT TATCTGCGGA ATTCCAGCTG    1169

AGCGCCGGTC CTACCATTAC CAGTTGGTCT GGTGTCGACG ACTCCTGGAG    1219

CCCGTCAGTA TCGGCGGAAT TCGCGGCCGG GCGCCAATGC ATTGGGCCCA    1269
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ATTCCGCCCT ATAGTGAGTC GTATTACAAT TCACTGGCCG TGTTTTACAA 1319

CCTCGTGACT GGGAAAACCC TGGCCTTACC CAAC 1353

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1423 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA to mRNA

(A) DESCRIPTION: galactosyl transferase, full coding sequence

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Sus scrofa

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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CGGGGGCCAT CCCCGAGCGC ACCCAGCTTC TGCCGATCAG GAGAAAATA                    49

ATG AAT GTC AAA GGA AGA GTG GTT CTG TCA ATG CTG CTT GTC                   91
Met Asn Val Lys Gly Arg Val Val Leu Ser Met Leu Leu Val
                5                   10

TCA ACT GTA ATG GTT GTG TTT TGG GAA TAC ATC AAC AGA AAC                  133
Ser Thr Val Met Val Val Phe Trp Glu Tyr Ile Asn Arg Asn
 15                  20                  25

CCA GAA GTT GGC AGC AGT GCT CAG AGG GGC TGG TGG TTT CCG                  175
Pro Glu Val Gly Ser Ser Ala Gln Arg Gly Trp Trp Phe Pro
      30                  35                  40

AGC TGG TTT AAC AAT GGG ACT CAC AGT TAC CAC GAA GAA GAA                  217
Ser Trp Phe Asn Asn Gly Thr His Ser Tyr His Glu Glu Glu
          45                  50                  55

GAC GCT ATA GGC AAC GAA AAG GAA CAA AGA AAA GAA GAC AAC                  259
Asp Ala Ile Gly Asn Glu Lys Glu Gln Arg Lys Glu Asp Asn
              60                  65                  70

AGA GGA GAG CTT CCG CTA GTG GAC TGG TTT AAT CCT GAG AAA                  301
Arg Gly Glu Leu Pro Leu Val Asp Trp Phe Asn Pro Glu Lys
                75                  80

CGC CCA GAG GTC GTG ACC ATA ACC AGA TGG AAG GCT CCA GTG                  343
Arg Pro Glu Val Val Thr Ile Thr Arg Trp Lys Ala Pro Val
 85                  90                  95

GTA TGG GAA GGC ACT TAC AAC AGA GCC GTC TTA GAT AAT TAT                  385
Val Trp Glu Gly Thr Tyr Asn Arg Ala Val Leu Asp Asn Tyr
     100                 105                 110

TAT GCC AAA CAG AAA ATT ACC GTG GGC TTG ACG GTT TTT GCT                  427
Tyr Ala Lys Gln Lys Ile Thr Val Gly Leu Thr Val Phe Ala
         115                 120                 125

GTC GGA AGA TAC ATT GAG CAT TAC TTG GAG GAG TTC TTA ATA                  469
Val Gly Arg Tyr Ile Glu His Tyr Leu Glu Glu Phe Leu Ile
             130                 135                 140

TCT GCA AAT ACA TAC TTC ATG GTT GGC CAC AAA GTC ATC TTT                  511
Ser Ala Asn Thr Tyr Phe Met Val Gly His Lys Val Ile Phe
                145                 150

TAC ATC ATG GTG GAT GAT ATC TCC AGG ATG CCT TTG ATA GAG                  553
Tyr Ile Met Val Asp Asp Ile Ser Arg Met Pro Leu Ile Glu
155                 160                 165
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CTG GGT CCT CTG CGT TCC TTT AAA GTG TTT GAG ATC AAG TCC     595
Leu Gly Pro Leu Arg Ser Phe Lys Val Phe Glu Ile Lys Ser
    170                 175                 180

GAG AAG AGG TGG CAA GAC ATC AGC ATG ATG CGC ATG AAG ACC     637
Glu Lys Arg Trp Gln Asp Ile Ser Met Met Arg Met Lys Thr
        185                 190                 195

ATC GGG GAG CAC ATC CTG GCC CAC ATC CAG CAC GAG GTG GAC     679
Ile Gly Glu His Ile Leu Ala His Ile Gln His Glu Val Asp
            200                 205                 210

TTC CTC TTC TGC ATT GAC GTG GAT CAG GTC TTC CAA AAC AAC     721
Phe Leu Phe Cys Ile Asp Val Asp Gln Val Phe Gln Asn Asn
                215                 220

TTT GGG GTG GAG ACC CTG GGC CAG TCG GTG GCT CAG CTA CAG     763
Phe Gly Val Glu Thr Leu Gly Gln Ser Val Ala Gln Leu Gln
225                 230                 235

GCC TGG TGG TAC AAG GCA CAT CCT GAC GAG TTC ACC TAC GAG     805
Ala Trp Trp Tyr Lys Ala His Pro Asp Glu Phe Thr Tyr Glu
    240                 245                 250

AGG CGG AAG GAG TCC GCA GCC TAC ATT CCG TTT GGC CAG GGG     847
Arg Arg Lys Glu Ser Ala Ala Tyr Ile Pro Phe Gly Gln Gly
        255                 260                 265

GAT TTT TAT TAC CAC GCA GCC ATT TTT GGG GGA ACA CCC ACT     889
Asp Phe Tyr Tyr His Ala Ala Ile Phe Gly Gly Thr Pro Thr
            270                 275                 280

CAG GTT CTA AAC ATC ACT CAG GAG TGC TTC AAG GGA ATC CTC     931
Gln Val Leu Asn Ile Thr Gln Glu Cys Phe Lys Gly Ile Leu
                285                 290

CAG GAC AAG GAA AAT GAC ATA GAA GCC GAG TGG CAT GAT GAA     973
Gln Asp Lys Glu Asn Asp Ile Glu Ala Glu Trp His Asp Glu
295                 300                 305

AGC CAT CTA AAC AAG TAT TTC CTT CTC AAC AAA CCC ACT AAA    1015
Ser His Leu Asn Lys Tyr Phe Leu Leu Asn Lys Pro Thr Lys
    310                 315                 320

ATC TTA TCC CCA GAA TAC TGC TGG GAT TAT CAT ATA GGC ATG    1057
Ile Leu Ser Pro Glu Tyr Cys Trp Asp Tyr His Ile Gly Met
        325                 330                 335

TCT GTG GAT ATT AGG ATT GTC AAG ATA GCT TGG CAG AAA AAA    1099
Ser Val Asp Ile Arg Ile Val Lys Ile Ala Trp Gln Lys Lys
            340                 345                 350

GAG TAT AAT TTG GTT AGA AAT AAC ATC TGACTTTAAA            1136
Glu Tyr Asn Leu Val Arg Asn Asn Ile
                355
```

TTGTGCCAGC AGTTTTCTGA ATTTGAAAGA GTATTACTCT GGCTACTTCC 1186

TCAGAGAAGT AGCACTTAAT TTTAACTTTT AAAAAAATAC TAACAAAATA 1236

CCAACACAGT AAGTACATAT TATTCTTCCT TGCAACTTTG AGCCTTGTCA 1286

AATGGGAGAA TGACTCTGTA GTAATCAGAT GTAAATTCCC AATGATTTCT 1336

TATCTGCGGA ATTCCAGCTG AGCGCCGGTC GCTACCATTA CCAGTTGGTC 1386

TGGTGTCGAC GACTCCTGGA GCCCGTCAGT ATCGGCG 1423

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 bases

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other Nucleic Acid

(A) DESCRIPTION: PCR primer αGT-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAATTCAAGC TTATGATCAC TATGCTTCAA 30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 bases

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other Nucleic Acid

(A) DESCRIPTION: PCR primer αGT-2

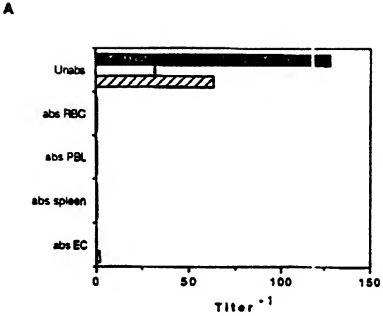
(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAATTCCTGC AGTCAGACAT TATTCTAAC 29

FIGURE 1


A
Unabs
abs RBC
abs PBL
abs spleen
abs EC
0
50
100
150
Titer -1

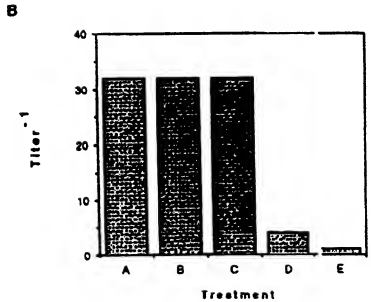

B
40
30
20
10
0
Titer - 1
A
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FIGURE 2

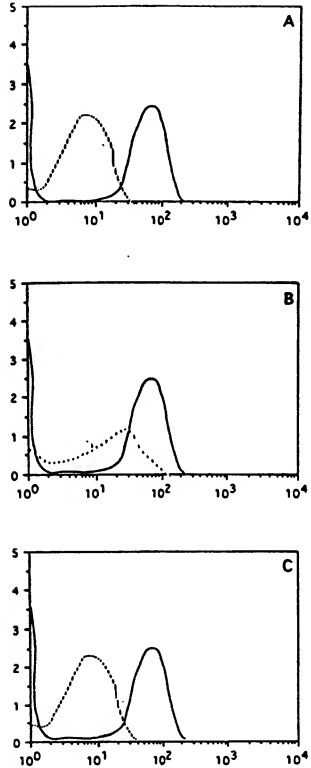

FIGURE 3

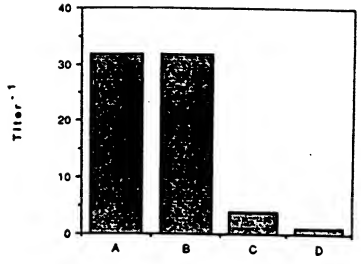

FIGURE 4

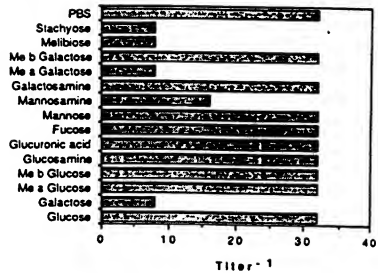

FIGURE 5

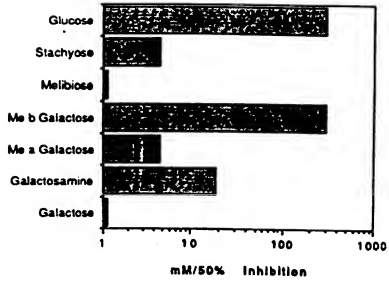

FIGURE 6

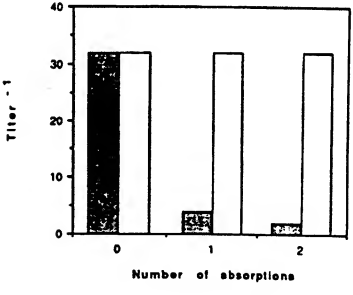

FIGURE 7